Passive Targeting of Doxorubicin with Polymer Coated Liposomes in Tumor Bearing Rats

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The purpose of this study was to reveal the effectiveness of the polymer coated liposomes as a carrier of the anticancer drug doxorubicin in intravenous administration. The size controlled doxorubicin-loaded liposomes (egg phosphatidylcholine : cholesterol=1 : 1 in molar ratio) were coated with hydrophilic polymers (polyvinyl alcohol; PVA and hydroxypropylmethylcellulose; HPMC) having a hydrophobic moiety in the molecules (PVA-R, HPMC-R). The existence of a thick polymer layer on the surface of the polymer coated liposomes was confirmed by measuring the change in particle size and the amount of polymer on the liposomal surface. The polymer coating effects on the tumor accumulation of the drug encapsulated in the liposomes were evaluated in Walker rat carcinoma 256 cell bearing rats. The doxorubicin-loaded liposomes coated with PVA-R and HPMC-R showed higher drug accumulation into the tumor site by prolonging the systemic circulation in tumor-bearing rats. The targeting efficiency of the polymer coated liposomes calculated with the total and tumorous clearance of the drug was *ca.* 5 times larger than that of non-coated liposomes. We ascertained that polymers having a hydrophobic moiety in the molecule such as PVA-R and HPMC-R are suitable materials for modifying the surfaces of the doxorubicin-loaded liposome to improve its targeting properties.

Key words　liposome; polymer coating; tumor accumulation; long circulating; doxorubicin

Liposomes have been regarded as a beneficial carrier system for parenteral application of a variety of drugs due to their biocompatible and biodegradable properties. It is also characteristic that the size of liposomes can be easily controlled to one suitable for injection. Injected liposomes as well as other colloidal particles, however, tend to be quickly removed by phagocytic cells existing primarily in liver and spleen as a result of their recognition as foreign bodies by the host's immune system.[1—3]

Many attempts such as surface modification of liposomes have been investigated in order to decrease the adsorption of blood components and consequently increase the blood circulation time. The surface modifications of liposomes with several biological materials including proteins, polysaccharides and glycolipids were found to improve the circulation time of liposomes injected by decreasing the uptake of liposomes in the reticuloendothelial system (RES).[4,5] Based on these findings, many efforts were undertaken to identify alternative materials for surface modification of liposomes, and further success could be obtained with the achievement of stealth liposomes using a phospholipid conjugated with a synthetic hydrophilic polymer, polyethyleneglycol.[6,7] It was also indicated that long circulation of the liposomes in blood resulted in a higher uptake of the drug in an implanted tumor.[8—10] In general, the blood vessels in tumor are inherently leaky because the basement membrane is discontinuous or absent,[11,12] so that liposomes with a prolonged circulation time may have a tendency to extravasate through discontinuous capillaries in the area of the leaky vascular system.[13,14]

Polymer coating is an alternative method to modify the surface of liposomes. We have reported the feasibility of the polymer coating of liposomes using modified hydrophilic polymers such as polyvinyl alcohol having a hydrophobic anchor at the end of a molecule (PVA-R). The formation of a steric coating layer on the surface of liposomes was confirmed with zeta potential and particle size measurement.[15] Such a thick coating layer was not observed in liposomes

coated with PVA having no anchoring moiety. It was also confirmed that the PVA-R coated liposomes, as well as PEGulated stealth liposomes, prolonged the circulation by decreasing their RES uptake in rats.[16,17] In comparing this polymer coating method with that for the PEGulated liposomes, the former is characteristic in coating the liposomes after preparation of a drug containing liposomal particles.[16]

In this paper, the passive targeting properties of the polymer coated liposomes were evaluated with the tumor accumulation of the antitumor drug, doxorubicin, entrapped in the liposomes by intravenously injecting the liposomal drug into tumor bearing rats. For coating the liposomal surface, two different hydrophilic polymers bearing hydrophobic anchors in the molecules, PVA-R and a modified hydroxypropylmethylcellulose (HPMC-R), were used. As the HPMC-R specially designed for this experiment has a very low substituting ratio with the hydrophobic group in a molecule, the formation of a steric coating layer with HPMC-R as well as with PVA-R was expected. The passive targeting effects of these polymer coated liposomes were evaluated with the targeting efficiency calculated by the total and tumorous clearance of the drug.

MATERIALS AND METHODS

Materials Egg phosphatidylcholine (COATSOME NC-10S) and L-α-dimilistoyl phosphatidylcholine (DMPC, COATSOME MC-4040) were purchased from Nippon Oil and Fats Co., Japan. Dicetyl phosphate (DCP) and cholesterol were obtained from Sigma Chemical Co. Doxorubicin hydrochloride was kindly donated by Kyowa Hakko Kogyo Co., Japan. Polyvinyl alcohol 205 (PVA) and its derivative bearing a hydrophobic anchor ($C_{16}H_{33}$–S–) at the terminal of the polymer molecule (PVA-R) was a gift from Kuraray Co., Japan. The degree of polymerization of PVA and PVA-R was 450 and 480, respectively. Hydroxypropylmethylcellulose (HPMC) and its derivative having a hydrophobic moiety

($C_{18}H_{37}$) in the molecules (HPMC-R) was supplied by Sankyo Chemical Co., Japan. The molecular weight and the substituting ratio of long alkyl chain (LA) of HPMC-R were *ca.* 200000 and 1.58 (mol/mol), respectively. All other chemicals were commercial products of reagent grade.

Preparation of the Doxorubicin Loaded Liposomes Coated with Polymers The size controlled liposomes (100 nm) were prepared from egg phosphatidylcholine and cholesterol (1 : 1, mole ratio) by the hydration–extrusion method. In brief, the lipid mixture (189.2 mg EPC, 94.8 mg cholesterol) was dissolved in a small amount of chloroform in a round-bottomed flask and dried in a rotary evaporator under a reduced pressure at 40 °C to form a thin lipid film on the flask. The film was dried in a vacuum overnight to ensure complete removal of the solvent. Multilamellar vesicles (MLVs) were formed with 3 ml 50 mM citric acid (pH 2.1) in saline in the flask with constant vortex mixing at 60 °C, followed by incubation for 1 h at 10 °C. MLVs were extruded 12 times by an extruder (Lipex Biomembranes, U.S.A.) equipped with the polycarbonate membrane (0.1 μm, Nucleopore®) to make small unilamellar liposomes (106.9±3.3 nm).

Both drug encapsulation and polymer coating were carried out at the same time according to a modified pH gradient (interior acidic) method previously reported.[16] In short, non-coated liposomes were prepared by adding 4 ml of 50 mM sodium carbonate in saline to the mixture of small-unilamellar liposomes (1 ml) and aqueous doxorubicin solution (1 ml). The resultant mixture was shaken for 10 min at 60 °C, followed by incubation for 1 h at 10 °C (non-coated liposomes). For preparation of the polymer coated liposomes, PVA-R or HPMC-R solution (2 ml) was mixed with an equal amount of 200 mM or 100 mM sodium carbonate in saline, respectively, and added to the liposomal suspension. The final concentration of PVA-R or HPMC-R in the suspension was 1 and 0.43%, respectively. The encapsulation efficiency of drug was almost 92% regardless of polymer coating as determined by the previously described method.[16]

In measuring the change in zeta potential of the liposomes by HPMC and HPMC-R coating, negatively charged liposomes 100 nm in diameter were prepared with DMPC, DCP and cholesterol of 7 : 3 : 1 in a molar ratio by the hydration method, followed by sonication (UR-200P, Tomy Seiko Co.). Polymer-coated liposomes were prepared by mixing the resultant liposomal suspension (1 ml) with an equal amount (1 ml) of polymer solution (0—1%), followed by incubation at 10 °C for 1 h.

Characterization of Polymer Coated Liposomes The particle size and distribution of the liposomes were measured with a dynamic light scattering method using LPA-300 equipment (Otsuka Electronics Co.). An aliquot of each liposomal suspension was diluted with a large amount of distilled water.

Zeta potential of the liposomes was evaluated with a zeta meter (Zeta Master, Malvern Instruments). To measure the zeta potential, a small amount of the liposomal suspension was diluted with a large amount of phosphate buffer solution at pH 7.4.

The coating amount of HPMC and HPMC-R was evaluated by a centrifugation method as previously reported.[15] In short, a small amount (0.3 ml) of the polymer coated liposomal suspensions was centrifuged at 75000 rpm for 120 min
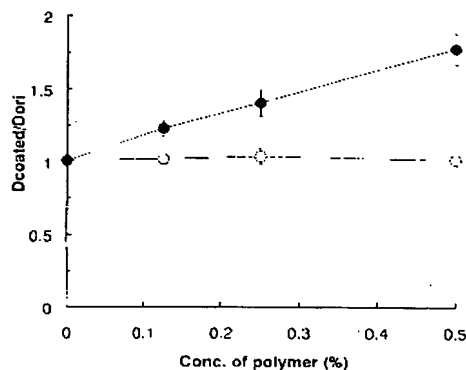

Fig. 1. Particle Size Change in Liposomes Following Coating with HPMC-R or HPMC (*n*=3)

Lipid composition of liposomes: EPC:Chol=5:5. ●, HPMC-R; ○, HPMC. D_{coated}, particle size of liposomes after coating process; D_{ori}, particle size of liposomes before coating.
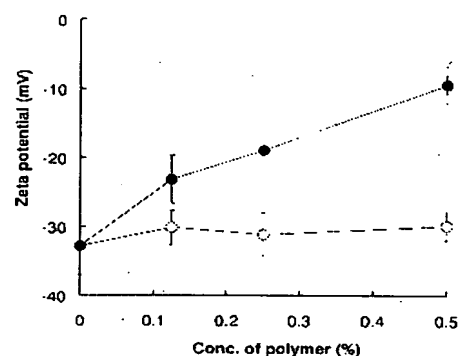

Fig. 2. Zeta Potential of Liposomes Coated with HPMC-R or HPMC (*n*=3)

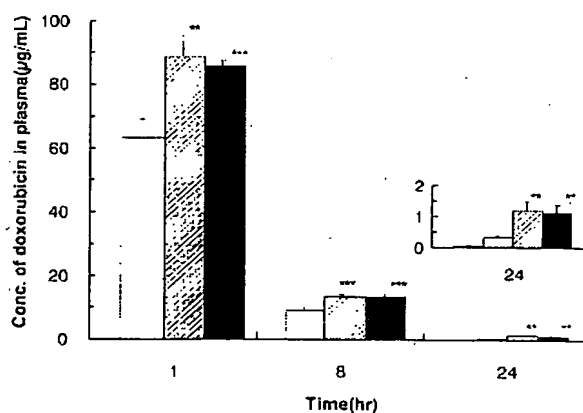Lipid composition of liposomes: DMPC:DCP:Chol=7:3:1. ●, HPMC-R; ○, HPMC.

Fig. 3. Doxorubicin Concentration in Plasma after I.V. Administration of Doxorubicin Solution or Various Liposomal Doxorubicins in Walker Rat Carcinoma Bearing Rats

Doxorubicin solution, □; non-coated-, ▣; HPMC-R-coated-, ▨; PVA-R-coated liposomal doxorubicin, ■. Lipid composition of liposomes: EPC:Chol=5:5. Dose of doxorubicin: 5 mg/kg weight. **$p<0.01$, ***$p<0.001$: significantly different from the levels for non-coated liposomal doxorubicin. Data at 24 h were shown in the inset with an extended scale.

(Hitachi CS-100), and the resultant supernatant was applied to the quantitative analysis of the polymers according to JPXIII. In short, the amount of isopropyl iodide, which corresponded to the hydroxypropoxyl group in cellulose of HPMC, was measured by a gas chromatograph. The amount of coating was calculated from the reduced polymer concentration in the solution after coating.

Plasma Pharmacokinetics and Tumor Accumulation of Doxorubicin in Tumor-Bearing Rats All animal experimental protocols were approved by the animal welfare commission of Gifu Pharmaceutical University. Male Wistar rats (body weight 220—250 g, 10 weeks old) purchased from Japan SLC, Inc. were maintained in standard housing. Walker rat carcinoma 256 (WRC256) cells were kindly donated by the Cancer Cell Repository (CCR) of Tohoku University. Tumor bearing rats were prepared by implanting the WRC256 cell suspension (1×10^7 cells) subcutaneously into the back (day 0), and allowing the tumor to grow for approximately 10 d. The tumor volume was calculated by the following equation

$$\text{tumor volume (cm}^3) = 0.5 \times a^2 \times b$$

where a is minor axis and b is major axis.

After confirming that the size of the grown tumor was ca. 30 ± 5 mm in length and width, rats were randomized into several groups (each group consisted of 4 to 6 rats, mean tumor volume of 11.4 ± 1.0 cm^3), and the doxorubicin solution or liposomal doxorubicin with or without polymer coating was injected intravenously into the animals via the tail vein at a dose of 5 mg doxorubicin/kg. At the time points of 1, 8, 24 and 48 h, the blood was collected from the retro-orbital sinus of the rats, then the tumor was immediately excised from the rats sacrificed, followed by homogenization with pH 8.0 citric buffer saline (5 ml/g tumor). The plasma separated from the blood components by centrifugation (3000 g, 10 min) was divided into two portions. The drug extracted into toluene/butanol (1 : 1, v/v) was subjected to HPLC assay with a fluorescence detector (Jasco FP-920, excitation 480 nm, emission 590 nm) in a manner previously reported.[16]

The targeting efficiency of the liposomal formulations to the tumor site was calculated by the following equations reported by Takakura and Hashida.[18] The total tumor accumulation ($X^{\infty}_{\text{tumor}}$) at infinite time after i.v. administration was calculated by assuming the tumor weight of 12 g.

$$\text{targeting efficiency} = X^{\infty}_{\text{tumor}}/\text{Dose} = CL_{\text{tumor}}/CL_{\text{total}}$$

$$CL_{\text{total}} = \text{Dose}/AUC$$

$$CL_{\text{tumor}} = X(t)_{\text{tumor}}/AUC_{0 \to t}$$

where CL_{total} and CL_{tumor} represent the total and tumorous clearance, respectively. $X(t)_{\text{tumor}}$ expresses the amount of tumor uptake at time t. $AUC_{0 \to t}$ represents area under the blood concentration–time curve from time 0 to t.

RESULTS

Characterization of Liposomes Coated with HPMC-R In a previous paper we demonstrated the formation of a thick polymer layer on the surface of PVA-R coated liposomes by measuring the particle size and amount of polymer on the li-

posomal surface.[15—17] A similar evaluation was carried out for the liposomes coated with hydroxypropylmethylcellulose (HPMC) and its derivative having hydrophobic anchors in the molecules (HPMC-R). The particle size of the liposomes coated with HPMC-R increased with increase in the polymer concentration used in the coating (Fig. 1). On the other hand, such an increase in particle size was not observed for the HPMC-coated liposomes irrespective of polymer concentration in the coating (Fig. 1). In measuring the amount of polymer coating the liposomal surface, the amount of HPMC-R coated liposome was much higher than that of the HPMC coated one. The coating amount of HPMC-R coated liposomes, for example, was 101.1 mg/lipid mmol which was 5.1-fold as large as that of HPMC coated ones at a polymer concentration of 0.5%. These results suggested formation of a thick coating layer with HPMC-R on the surface of the liposomes as well as PVA-R coated liposomes.

The zeta potential analysis of the polymer coated liposomes was carried out using the anionic liposomes composed of DMPC : DCP : cholesterol in a molar ratio of 7 : 3 : 1 (Fig. 2). The zeta potential of HPMC-R coated liposomes gradually reached zero with increasing polymer concentration in the coating process. The change in zeta potential of these HPMC-R coated liposomes confirmed the formation of the thick polymer layer on the surface. No significant change in zeta potential was observed for the HPMC coated liposomes (Fig. 2), however, which was attributed to the formation of a thin coating layer formed on the liposomal surface. These results corresponded to the results observed for the PVA and PVA-R coated liposomes.[15]

Passive Targeting with Polymer-Coated Liposomes In previous papers,[16,17] we reported the prolonged circulation of PVA-R coated liposomes. It was also demonstrated that circulation of the liposomal doxorubicin was improved by using polymer coated liposomes.[16] To evaluate the passive targeting effects of these latter liposomes, the circulation of doxorubicin encapsulated into the PVA-R and HPMC-R coated liposomes was measured in rats bearing Walker rat carcinoma 256 cells on their back. Figure 3 shows the drug concentration in plasma up to 24 h after injection with the various liposomal formulations of doxorubicin. Significantly higher drug retention was observed for PVA-R and HPMC-R coated liposomes compared with non-coated ones at each sampling time. It was also found that the drug circulation with the HPMC-R coated liposomes was almost the same as that observed with the PVA-R coated ones. The prolongation effect of drug circulation was not observed for the HPMC coated liposomes; the circulation with these was almost the same as that observed for the non-coated liposomal drug (data not shown).

When the doxorubicin concentration in the solid tumor implanted on the back of rats was measured after intravenous injections of the liposomal systems, an improvement in drug accumulation was observed for the polymer-coated liposomal formulations (Fig. 4). The drug levels in tumor after intravenous injection of doxorubicin solution, which showed a peak value of ca. 3.34μg/g tumor at 1 h post administration, decreased rapidly and became only a trace at 24 h. In the case of non-coated liposomal formulation the drug concentration in the tumor site also showed the peak value at 1 h and decreased gradually as time passed. On the other hand, in ap-
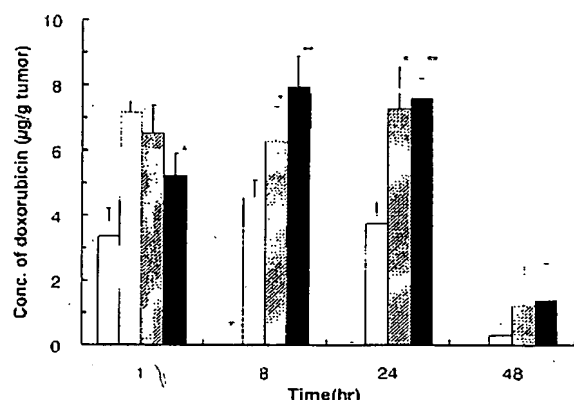
Fig. 4. Drug Accumulation in Tumor after I.V. Administration of Doxorubicin Solution or Various Liposomal Doxorubicins in Walker Rat Carcinoma Bearing Rats

Doxorubicin solution, □; non-coated-, ■; HPMC-R-coated-, ▨; PVA-R-coated liposomal doxorubicin, ■. Lipid composition of liposomes: EPC:Chol=5:5. Dose of doxorubicin: 5 mg/kg weight. $*p<0.05$, $**p<0.01$: significantly different from the levels for non-coated liposomal doxorubicin.

Table 1. Pharmacokinetic Parameters of Liposomal Doxorubicins Injected Intravenously in Walker Rat Carcinoma Bearing Rats

	Polymers		
	Non-coated	PVA-R	HPMC-R
AUC^{0-48h}_{plasma} ($\mu g \cdot h/ml$)	391.2	555.2	567.8
CL_{total} (ml/h)	3.2	2.25	2.20
AUC^{0-48h}_{tumor} ($\mu g \cdot h/g$)	159.6	280.8	258.1
CL_{tumor} (ml/h)	9.24×10^{-3}	2.99×10^{-2}	2.58×10^{-2}
Targeting efficiency	2.89×10^{-3}	1.33×10^{-2}	1.17×10^{-2}
Total tumor accumulation (μg)	3.6	16.6	14.6

Lipid composition of liposomes: EPC:Chol=5:5. Dose of doxorubicin: 5 mg/kg weight.

plying the PVA-R or HPMC-R coated liposomes, the higher drug concentration remained until 24 h after injection probably because of their prolonged circulation. As a result, almost a double tumor concentration was observed for PVA-R and HPMC-R coated liposomal systems compared with the non-coated system 24 h after injection. Even 48 h after injection, considerable amounts of doxorubicin were detected for the polymer coated liposomal systems.

The effectiveness of the colloidal carrier systems in passive tumor targeting of the drug can be evaluated by the pharmacokinetic parameters such as the area under the drug concentration (AUC), the clearance and the targeting efficiency, which have been successfully used for the evaluation of macromolecular carriers by Takakura and Hashida.[18] The tumor accumulation of drug was evaluated by the area under the drug concentration in the tumor–time curve from 0 to 48 h post injections (AUC_{tumor}). The values of PVA-R and HPMC-R coated liposomes were 1.76- or 1.62-fold as large as those of non-coated ones, respectively, confirming the improvement in drug accumulation in a tumor site by use of the polymer-coated liposomes (Table 1). Table 1 also lists the total clearance (CL_{total}) and tumor uptake clearance (CL_{tumor}). The lower CL_{total} for the polymer coated liposomes can be attributed to the reduction in their RES uptake.[17] The higher

CL_{tumor} of the polymer coated liposomes suggests their higher affinity to the tumor cell or extracellular spaces and/or the increased stability of liposome by polymer coating. The targeting efficiency calculated from the values of CL_{tumor} and CL_{total} revealed $ca.$ a 5 times higher efficacy for the polymer coated liposomes compared with non-coated liposomes. The total tumor accumulation of drug was also calculated for these liposomal systems (Table 1). On the other hand, the CL_{tumor} of drug solution was not calculated because the drug levels in tumor at 24 and 48 h post injection were too low to be detected. The AUC value in plasma and in tumor for free drug administration was 1.98 μg h/ml and 20.3 μg h/g, respectively.

DISCUSSION

It is well accepted that the polymer layer formed on the liposomal surface may effectively protect the liposomes from interacting with plasma proteins in the blood, leading to a reduction in RES uptake and prolonged circulating time in blood.[19] We have reported that the steric polymer layer formed with PVA-R on the surface of liposomes effectively prevents the RES uptake of the liposomes, leading to the prolonged circulation in tumor-free rats. The prolonged circulation of doxorubicin was achieved with HPMC-R coated liposomes as well as PVA-R coated ones in tumor bearing rats. As such an improvement was not observed for the HPMC coated liposomal system, it was confirmed that the thick polymer layer tightly formed with PVA-R and HPMC-R on the surface of liposomes played an important role in prolonging drug circulation, which was probably attributed to a lesser uptake of liposomes in the reticuloendothelial system.

In both polymer coated liposomes, the retention of drug at 8 and 24 h was lower than expected from the data on retention of the carriers. For example, the retained dose % of PVA-R coated liposome carriers in blood measured in normal rats was 29.1% at 8 h and 11.8% at 24 h, respectively, while that of drug encapsulated in the PVA-R coated liposomes was 14.7% at 8 h and 1.1% at 24 h. The difference in the residual % of carriers and drug in blood of the tumor bearing rats could be explained by both the leakage of drug from the carriers in the blood circulation and the distribution of liposomal drug into the tumor.

In general, the size controlled liposomes with a long circulating property could usually extravasate into the extracellular spaces of the solid tumor through the leaky endothelium.[20] In case of the lack of rapid elimination by the lymphatic system, they could remain there for a long time and release their contents gradually. A possible explanation for the increased extravasation through the endothelial layer in tumors is a result of the enhanced permeability of the endothelial barriers in the newly vascularized tumors.[9] The pathophysiological characteristics of solid tumors described above is a so-called enhanced permeability and retention (EPR) effect, which enables the passive targeting of anti-cancer drug to solid tumors with the macromolecular drug delivery systems.[12]

The increased tumor accumulation of the liposomal doxorubicin including the polymer-coated ones may be explained by the EPR effects. The higher tumor accumulation of the polymer-coated system than the non-coated one indi-

cated an improved EPR effect by polymer coating. The permeating property might not be responsible for the different EPR effects of these particulate systems, because the particle size of the polymer coated liposomes was rather larger than non-coated ones. Thus, the enhanced EPR effects of the polymer coated liposomal systems over the non-coated ones were attributed to their higher retention. A possible explanation for the improved retention was the higher affinity of the polymer coated liposomes to the tumor cells or extracellular spaces and/or their increased stability after passing the endothelial layer. Although we have demonstrated the improved stability of the PVA-R coated liposomes in the presence of serum *in vitro*,[15] so far no evidence has been observed for these factors in the tumor cells.

In physiological pharmacokinetic analysis, the enhanced EPR effects of the polymer coated liposomes were clearly characterized by both the decreased total body clearance (CL_{total}) and the increased tumor uptake clearance (CL_{tumor}). The decreased CL_{total} observed for the polymer coated systems, which clearly showed their passive targeting function, were mainly attributable to the decreased RES uptake. The extent of increase in the CL_{tumor} of the liposomal system by polymer coating (*ca.* 3 times) was much higher than that expected with the change in CL_{total} (Table 1).

In conclusion, polymer coating is effective in increasing the tumor accumulation of the doxorubicin-loaded liposomes with increase in the drug circulating time in tumor bearing rats. In a previous paper,[16] we demonstrated that the polymer coated liposomal systems can reduce the drug distribution to the heart, which can lead to reduction in its severe cardiac toxicity. The therapeutic evaluation of these polymer coated liposomal doxorubicin systems is in progress with tumor bearing mice and will be reported in a subsecuent paper.[21]

REFERENCES

1) Poste G., Bucana C., Raz A., Bugelski P., Kirsh R., Fidler I. J., *Cancer Res.*, **42**, 1412—1422 (1982).
2) Senior J. H., *Crit. Rev. Ther. Drug Carrier Syst.*, **3**, 123—193 (1987).
3) Papahadjopoulos D., Gabizon A., *Ann. N.Y. Acad. Sci.*, **507**, 64—74 (1987).
4) Lasic D. D., Papahadjopoulos D. (ed.), "Medical Application of Liposomes," Elsevier, Amsterdam, 1998.
5) Woodle M. C., Lasic D. D., *Biochim. Biophys. Acta*, **1113**, 171—199 (1992).
6) Klibanov A. L., Maruyama K., Torchilin V. P., Huang L., *FEBS Lett.*, **268**, 235—237 (1990).
7) Allen T. M., Hansen C., Martin F., Redemann C., Yau-Young A., *Biochim. Biophys. Acta*, **1066**, 29—36 (1991).
8) Poznansky M., Juliano R. L., *Pharmacol. Rev.*, **36**, 277—336 (1984).
9) Gabizon A., Papahadjopoulos D., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6949—6953 (1988).
10) Gabizon A., Shiota R., Papahadjopoulos D., *J. Natl. Cancer Inst.*, **81**, 1484—1488 (1989).
11) Dvorak H. F., Nagy J. A., Dvorak J. T., Dvorak A. M., *Am. J. Pathol.*, **133**, 95—109 (1988).
12) Maeda H., Seymour L. W., Miyamoto Y., *Bioconjug. Chem.*, **3**, 351—362 (1992).
13) Gabizon A., Price D. C., Huberty J., Bresalier R. S., Papahadjopoulos D., *Can. Res.*, **50**, 6371—6378 (1990).
14) Papahadjopoulos D., Allen T. M., Gabizon A., Mayhew E., Matthay K., Huang S. K., Lee K. D., Woodle M. C., Lasic D. D., Redemann C., Martin F. J., *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11460—11464 (1991).
15) Takeuchi H., Yamamoto H., Toyoda T., Toyobuku H., Hino T., Kawashima Y., *Int. J. Pharm.*, **164**, 103—111 (1998).
16) Takeuchi H., Kojima H., Toyoda T., Yamamoto H., Hino T., Kawashima Y., *Eur. J. Pharm. Biopharm.*, **48**, 123—129 (1999).
17) Takeuchi H., Kojima H., Yamamoto H., Kawashima Y., *J. Control. Rel.*, **68**, 195—205 (2000).
18) Takakura Y., Hashida M., *Pharm. Res.*, **13**, 820—831 (1996).
19) Senior J., Delgado C., Fisher D., Tilcock C., Gregoriadis G., *Biochim. Biophys. Acta*, **1062**, 77—82 (1991).
20) Ning Z. W., Daphne D., Tracy L. R., David N., Richard A. W., Mark W. D., *Can. Res.*, **53**, 3765—3770 (1993).
21) Takeuchi H., Kojima H., Toyoda T., Yamamoto H., Hino T., Kawashima Y., *J. Pharm. Pharmacol.*, submitted.